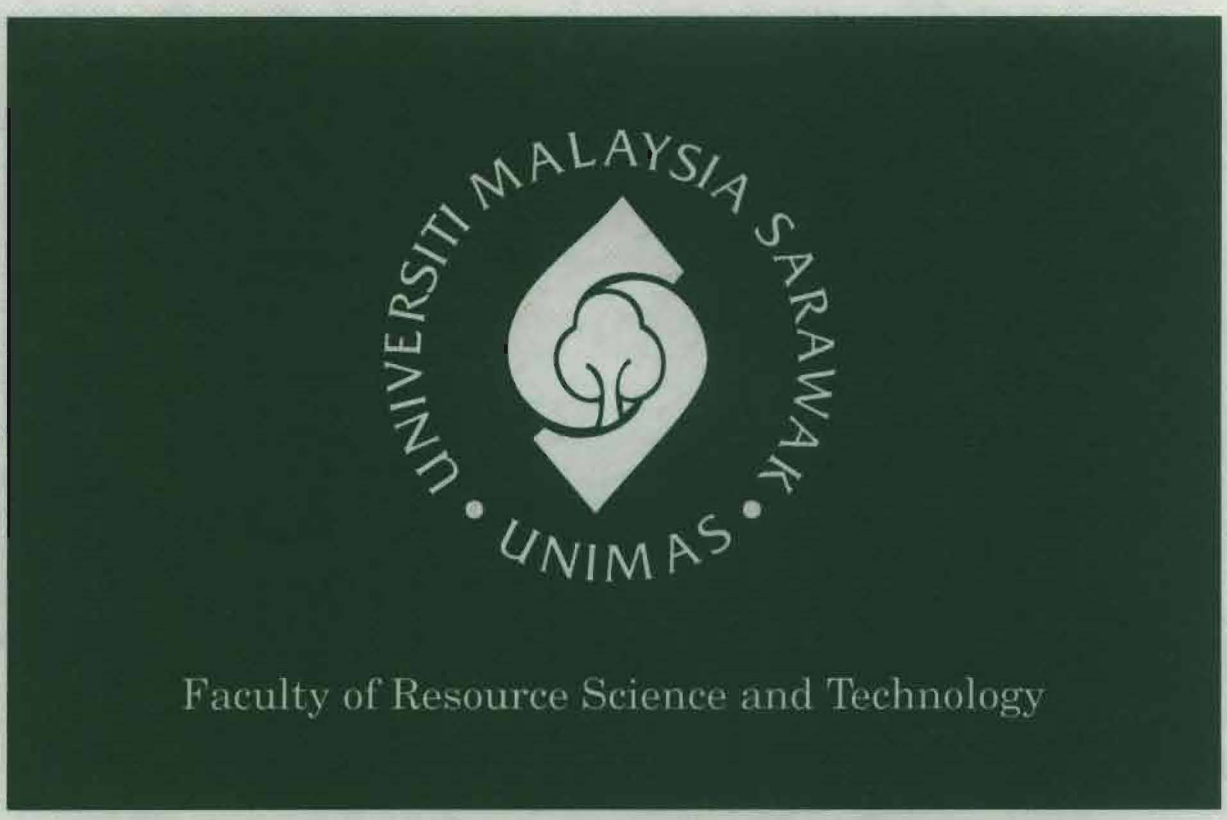

OPTIMIZING CLEAVED AMPLIFIED POLYMORPHIC SEQUENCE MOLECULAR TECHNIQUE IN DEVELOPING *Eusideroxylon zwageri* Teijsm & Binn. AND *Potoxylon melagangai* Kosterm. (BORNEO IRONWOOD) MOLECULAR MARKER

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Bachelor of Science with Honours
(Resource Biotechnology)
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ABSTRACT

One of our most valuable timber trees is the Borneo Ironwood. More fondly known as Belian by Sarawakians, it is one of the most renowned timbers of Borneo. This tree comes from the family Lauraceae. In general, there are two species of Belian that can be found in Southeast Asia: *Eusideroxylon zwageri* and *Potoxylon melagangai.* Both species possess similar phenotypic characteristics making it hard to differentiate between the two species. Unfortunately, the extraction of genomic DNA from both species using modified CTAB extraction approach was unsuccessful. However, three samples from each of the species were kindly provided by Tan Sia Hong and the DNA samples were then amplified using PCR utilizing chloroplast DNA (cpDNA) primers, *rbcL* and *rpoC* primers. However, only *P. melagangai* DNA was successfully amplified using the *rbcL* primer. None of the *E. zwageri* managed to be amplified using either *rbcL* or *rpoC* primers. Thus, the development of molecular marker to differentiate *P. melagangai* and *E. zwageri* at genomic level was unsuccessful.

Key words: CTAB extraction approach, *rbcL* and *rpoC* primers, PCR

ABSTRAK

Borneo Ironwood merupakan salah satu di antara kayu balak yang tinggi nilainya. Dikenali oleh orang tempatan sebagai Belian, ia merupakan salah satu spesies pokok balak yang terkenal di Kepulauan Borneo. Spesies pokok ini berasal dari famili Lauraceae. Umumnya, terdapat dua spesies Belian yang boleh dijumpai di Asia Tenggara: Eusideroxylon zwageri dan Potoxylon melagangai. Kedua-dua spesies mempunyai ciri-ciri fizikal yang hampir sama menjadikan ia susah untuk membezakan kedua-dua spesies ini. Tetapi, pengekstrakan DNA genomik kedua-dua spesies yang menggunakan kaedah pengekstrakan CTAB tidak berjaya. Justeru itu, tiga sampel daripada setiap spesies Belian telah disediakan dengan budi bicara Tan Sia Hong. Kemudian, sampel DNA itu menjalani PCR menggunakan primer rbcL dan rpoC untuk mengamplifikasikan jumlah DNA yang sedia ada. Tetapi, hanya DNA dari P. melagangai berjaya diamplifikasi menggunakan primer rbcL. Tiada satu pun DNA daripada E. zwageri yang berjaya diamplifikasi. Oleh itu, penanda molekul untuk membezakan P. melagangai dan E. zwageri di tahap genomik tidak berjaya dihasilkan.

Kata kunci: Kaedah pengekstrakan CTAB, primer rbcL dan rpoC, PCR

CHAPTER 1

INTRODUCTION

Sarawak is blessed with abundance of natural resources such as petroleum, liquefied natural gas (LNG) and most importantly precious and unique timber trees. Sarawak's forests are the state's most important resource and asset, producing timber and a large number of forest products. The production of approximately 9 to 10 million cubic metres of logs annually makes Sarawak as one of the world's largest exporters of tropical hardwood timber and making up about 40% of the state's real Gross Domestic Product (GDP) contributed by the primary sectors, which include forestry, agriculture and mining.

One of our most valuable timber trees is the Borneo Ironwood or more fondly known as Belian by Sarawakians. It is undeniably one of the most renowned timbers of Borneo. This tree comes from the family Lauraceae. In general, there are two species of Belian that can be found in Southeast Asia: *Eusideroxylon zwageri* and *Potoxylon melagangai.* Both species possess similar phenotypic characteristics making it hard to differentiate between the two species.

A study done by Kostermans in 1979 had shown that *Eusideroxylon zwageri* is a very distinct species from *Potoxylon melagangai* and the study also proven that both species though possess almost the same morphology, are indeed different at the molecular level.

Besides that, the studies reported by Kostermans (1979), Soerjanegara *et al.* (1994) and Teo (1998) collectively reported the similarity of the two species of Belian. The obvious likeness between the two is regarding the morphological characters in which they share the same shape and type of fruits, almost the same shape of leaves and trunk. Therefore, it is hard to differentiate the Belian based on their physical characters.

According to Paul & Max (1993), Belian has been considered as an endangered species mostly due to overexploitation of human for export trade. In 1998, the International Union for the Conservation of Nature and Natural Resources (IUCN) listed Borneo Ironwood under the 'Vulnerable' category in their red list. The criteria that befitted a taxon is 'Vulnerable' is that when the species is not Critically Endangered or Endangered but is facing a high risk of extinction in the wild in the medium-term future.

Traditional method for verification of species identity is based on the morphology and anatomy of the plant. However, this type of scientific yet impractical, in the case of Belian, verification method may give inconclusive results. This is mainly due to external influences both environmental and developmental, influencing the morphology and the close phenotype resemblance among clones from selection response for similar characteristics (Chong *et al.*, 1995).

The tool of modern biotechnology is considered to be the most applicable method for plant diversity characterization and also a major role in assisting plant

conservation programmes. Timber trees such as *E. zwageri* and *P. melagangai* are examples of economically important plant species.

Therefore, it is critical to consider the relationship between conservation and utilization of the trees (Benson, 1999). One way to achieve both conservation and utilization of the valuable trees is by utilizing marker techniques. For that reason, the development of molecular marker for *Eusideroxylon* spp. will enable the assessment of plant diversity at the genomic level (Ayad *et al.*, 1997).

The objectives of the study are;

1) To extract good quality total genomic DNA from *E. zwageri* and *P. melagangai* for subsequent DNA analysis.
2) To develop molecular marker for *E. zwageri* and *P. melagangai* via PCR-based technique using cpDNA primer.
3) To differentiate *E. zwageri* and *P. melagangai* based from the molecular aspect of the species.

CHAPTER II

LITERATURE REVIEW

Eusideroxylon zwageri Teijsm & Binn.

E. zwageri is one of the species of timber trees that is well known for durability and high economical value. This species derives from the family Lauraceae and are mostly found in riverine or alluvial forest in Brunei Darussalam, Indonesia (Jawa; Kalimantan; Sumatera), Malaysia (Sabah; Sarawak) and the Philippines. The riverine forest in the Samunsam Wildlife Sanctuary was once selectively harvested for ironwood or belian (*E. zwageri*) prior to the constitution of the sanctuary. Figure 2.1, exhibit the vegetation of the Samunsam Wildlife Sanctuary.

Eusideroxylon zwageri is generally known as belian batu, ironwood or billian (English). Fully grown tree of *E. zwageri* is medium to large-sized reaching a height of 40-50 m with its maximum girth of approximately 4 m. *E. zwageri* is suitable in marine construction for example to build wharfs, docks, dams , ships and as piling. Besides that, the long raised plank walkway of the Niah caves is made of belian as it can resists rot and termite attack far longer than other woods (Hazebroek and Abang Morshidi, 2001).

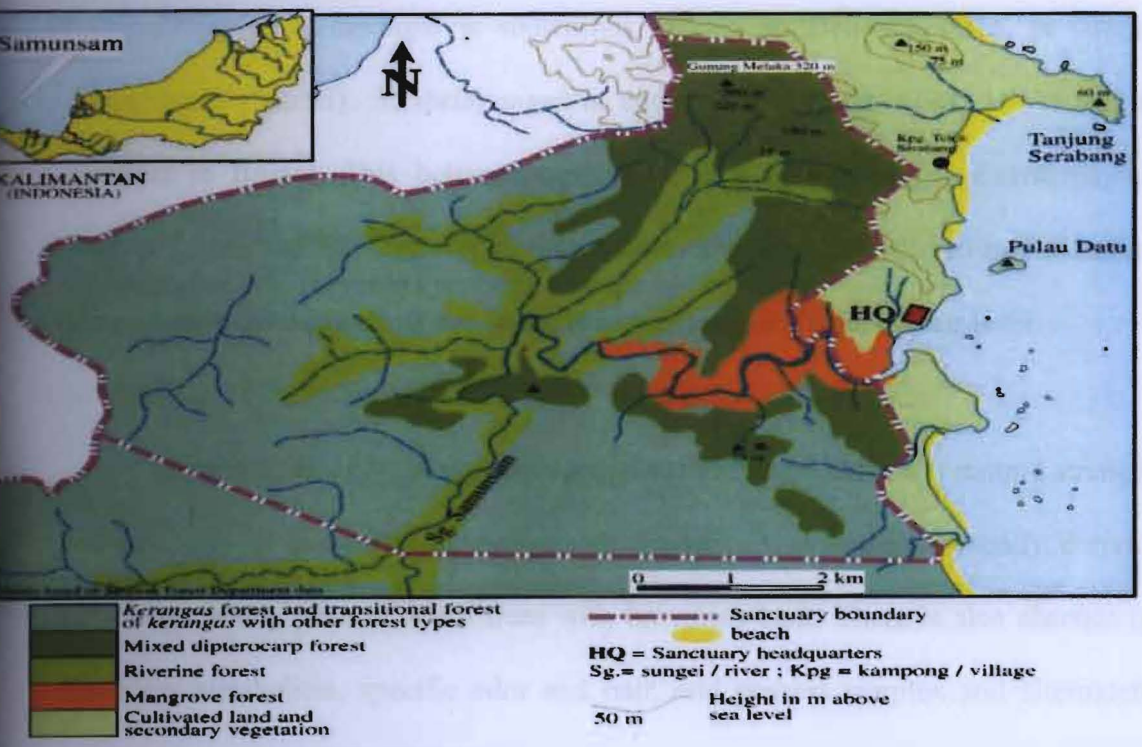

Figure 2.1. Vegetation of the Samunsam Wildlife Sanctuary (adapted from Hazebroek and Abang Morshidi, 2001)

2 ***Potoxylon melagangai Kosterm.***

Figure 2.2. The leaves of *Potoxylon melagangai*

Potoxylon melagangai is sometimes known as 'Belian kebuau' or simply 'Malagangai' (Brunei). *P. melagangai* is endemic to Borneo (Teo, 1998) and is prevalent in Brunei. This belian species was once referred to as *Eusideroxylon zwageri* Teijsm and Binn. However, the species name was later altered to the current name because the two species are found to be different at the molecular level.

Morphologically, *P. melagangai* wood is lighter and has lower natural strength and durability as compared *E. zwageri*. Moreover, *P. melagangai* usually derived from large woody trees or small trees with brownish bark. There is also absence of secondary metabolites, specific odor and hair, and possess simplex and alternately arranged leaves (Jarvie *et al.*, 1996).

In addition to that, a study conducted by Awee (2002) on *P. melagangai* had shown different morphological characteristics of *P. melagangai* and *E. zwageri*. Awee (2002) had reported that *P. melagangai* has the average of 680-860/ mm^2 isodiametric epidermis cells, the existence of paracytic stomata located on the adaxial and abaxial leaf surface with stomatic index of 0.013-0.015 and 0.17-0.279, respectively. There were no multiseriate rays present just uniseriate and biseriate rays. Lastly, the parenchyma cells were present in the form of aliform and confluent types.

From the construction aspect, *P. melagangai* is an alternative to the more durable *E. zwageri* or belian batu. *P. melagangai* is used in house and bridge construction. Apart from that, it is also used for making furniture, roof shingles and boat construction. Products made from this timber tree usually can last for up to forty to fifty years.

CTAB extraction method

In molecular marker studies, extracting DNA from a large number of plant accessions is quite a problem in plants that have high levels of polysaccharides and secondary metabolites (Pandey *et al.*, 1996). Therefore, a simple, rapid, economical, and effective DNA extraction method is highly desirable. High-quality DNA is essential in many studies of PCR-based markers, but storage of plant material collected in the field seldom ensures that quality DNA can be obtained.

CTAB DNA extraction method was introduced in 1987 by Doyle and Doyle. However, the high level of polysaccharides and secondary metabolites found in Belian makes extraction of high quality DNA quite a hard feat. Lodhi *et al.* (1994) stated that the contaminants in the plants will cause some problems during DNA purification where the samples will be viscous which makes the restriction endonucleases unable to restrict the DNA and consequently fail to amplify the targeted DNA.

Therefore, one way to eliminate polyphenols in the sample is by adding in PVP. The plant material must be kept frozen, in order to reduce the presence of polyphenolic compounds, before the extraction method is carried out. PVP functions to eliminate polyphenols by forming complex hydrogen bond with them. The PVP-polyphenol complex can then be separated from the DNA by centrifugation.

PCR-based molecular marker

Molecular markers are important tools of modern plant breeding. They are used for identification and characterization of germplasm, assessing DNA polymorphism and genetic diversity studies. Some useful methods in molecular marker technologies are restriction fragment length polymorphism (RFLP) and several PCR-based techniques, such as random amplified polymorphic DNA (RAPD), sequence-tagged site analysis (STS), microsatellite analysis, and amplified fragment length polymorphism (AFLP).

Molecular markers that are used to assess DNA polymorphism can be categorized into two type namely the hybridization-based markers and PCR-based markers. Hybridization-based markers involve the hybridization of the restriction enzyme-digested DNA to a labeled probe that is a fragment of DNA of known origin or sequence. On the other hand, PCR-based markers require the help of specifically or randomly chosen oligonucleotide sequences, better known as primers, and a thermostable DNA polymerase enzyme (*Taq* DNA polymerase), to amplify a particular DNA sequences or loci *in vitro*. The purpose of choosing the primer sequences are to allow base-specific binding to the template in reverse direction.

Molecular markers are considered as an important tool for plant genome analysis for numerous field of biology such as taxonomy, physiology and genetic engineering. DNA markers give scientists a more objective approach to analyze data compared to the traditional phenotypic markers.

Moreover, molecular marker will enable conservation strategies and at the same time allow economically significant species to be both utilized and protected. This is a major issue in countries that are rich in biodiversity and for which there is the need for populations to realize the economic potential of their abundance of biological resources and simultaneously preserve them for future generations.

Cleaved amplified polymorphic sequences (CAPS)

Since the discovery of PCR method in 1983 by Cary Mullis, it has gained rapid recognition and has become one of the most frequently used techniques to undertake molecular biology studies. The reasons for this overwhelming response to PCR are because it is inexpensive, fast and an easy method to produce more than 10 million copies of a targeted DNA sequences from small amount of starting materials (Bah Rem, 2005).

The polymerase chain reaction (PCR) is a method for oligonucleotide primer directed enzymatic amplification of a specific DNA sequence of interest. A prerequisite for amplifying a sequence using PCR is to have known, unique sequences flanking the segment of DNA to be amplified so that specific oligonucleotides can be obtained. It is not necessary to know anything about the intervening sequence between the primers.

Williams *et al.* (1991) and Arnold *et al.* (1991) came up with the idea to create molecular markers by digesting PCR products with restriction enzymes. CAPS or PCR-RFLP markers are generated in two steps. Firstly, a defined DNA sequence is amplified using a sequence specific primer pair. The product will undergo the second

step, which is the digestion with restriction enzyme, usually a four-base recognition specificity. The digestion of PCR products by the restriction enzyme and specific primers will generate polymorphic patterns. The PCR-based molecular markers have shown to detect high level of genetic polymorphism, which is the simultaneous occurrence of a feature in the same population of two or more discontinuous variants or genotypes, within species.

This marker technique differs from conventional RFLP analysis because CAPS does not require radioactivity or blotting step, but instead exhibits all the attractive attributes of PCR-based techniques. This advantage will reduce the risk of exposure to radioactive compound. Thus reducing the harm posed to the researcher.

Molecular marker developed using Cleaved Amplified Polymorphic Sequence technique is codominant, which is a desirable property in a molecular marker. The possibility to distinguish homozygous and heterozygous states makes the procedure attractive for mapping purposes (Drenkard *et al.*, 1998)

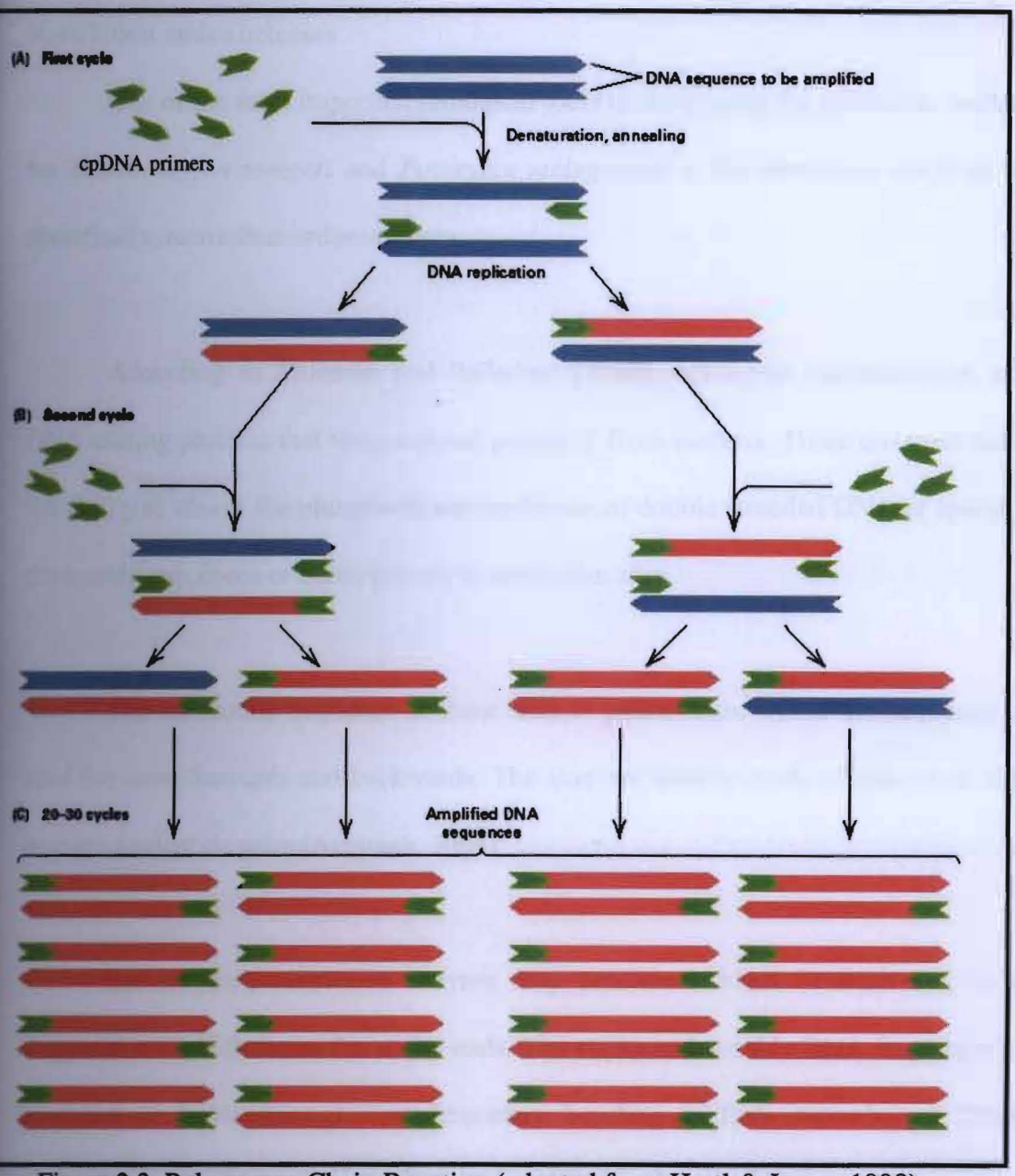

Figure 2.3. Polymerase Chain Reaction (adapted from Hartl & Jones, 1998)

Restriction endonucleases

One of the most important biological tools in developing the molecular marker for *Eusideroxylon zwageri* and *Potoxylon melagangai* is the restriction enzymes or specifically, restriction endonucleases.

According to Thieman and Palladino (2004), restriction endonucleases are DNA-cutting proteins that were derived primarily from bacteria. These enzymes have the ability to cleave the phosphodiester backbone of double stranded DNA at specific nucleotide sequences or better known as restriction sites.

The nucleotide sequence at these sites is palindromic where the sequence is read the same forwards and backwards. The sites are usually made of four, five, six, or eight nucleotide pairs (Acquaah, 2004).

Besides that, restriction enzyme may produce a blunt or flush cut, or a staggered cut which forms the sticky ends. The sticky ends enable DNA fragments to re-anneal or ligate through complementary bonding to form recombinant DNA molecules, when appropriate conditions are provided after the cleavage.

Agarose gel electrophoresis

Electrophoresis is the biochemical technique used for separating compounds in an electrical gradient (Acquaah, 2004). The compounds are separated based on the differences in molecular or physical structure and chemical properties. It is a common technique in molecular biology in order to separate and visualize DNA fragments

digested by restriction enzymes and subsequently analyze the pattern of fragments to come up with restriction map.

Agarose gel electrophoresis is conducted in agarose gel support matrix. As agarose cools, it solidifies forming horizontal semi-solid gel, in the electrophoresis plastic tray, containing small pores through which DNA fragments will travel (Thieman and Palladino, 2004). When running a gel, it must be submerged in a buffer solution that will conduct electricity. DNA samples are then loaded into small holes in the gel, which are the wells. Then, electric current is applied through electrodes at opposite ends of the gel.

The basis of separating DNA by electrophoresis is that DNA migrates in the gel according to its charge and size of the molecule that makes the migration distance of the molecule inversely proportional to the size of a DNA fragment.

However, the migrations of the DNA samples are impossible to track as the nucleic acids are often colorless. Therefore, gel visualization techniques must be utilized after gel electrophoresis. After the desired time of electrophoresis, DNA in the gel can be stained with dyes such as ethidium bromide that is able to intercalate between the base pairs of DNA. These dyes are visible when viewed ultraviolet (UV) light. When the gel has been stained with ethidium bromide and viewed under UV light, there will be bands visible on the gel. The fragments are then arranged in the correct order to create a restriction site map (Thieman and Palladino, 2004).

CHAPTER III

MATERIALS AND METHODS

Plant materials

The leaf of a plant has been known to be the best part to obtain genomic DNA required for DNA analysis. The total genomic DNA was extracted from leaves obtained from *E. zwageri* and *P. melagangai* trees maintained in FRST green house. The leaves were placed in a dark room for three days prior to DNA extraction to reduce polysaccharides content. Physical sterilization was performed to get rid of all contaminants on the surface of the leaves.

Materials

Liquid nitrogen

Mortar, pestle, scalpels

1 M Tris HCl pH 8.0

0.5 M EDTA pH 8.0

CTAB extraction buffer (EB)

100 mM Tris HCl pH 8.0

20 mM EDTA pH 8.0

1.4 M NaCl

2% Cetyl trimethy ammonium bromide (CTAB) (SIGMA H-5882)

1% PVP (Polyvinylpyrrolidone, Mr 40000)

0.2% β-mercaptoethanol

CIA: Chloroform-isoamyl alcohol (24:1)

Isopropanol

Wash buffer

76% ethanol

10 mM ammonium acetate

TE buffer pH 8.0

10 mM Tris HCl pH 8.0

1 mM EDTA pH 8.0

Water bath or incubator (37°C)

PCI: Phenol:Chloroform:Isoamylalcohol (25:24:1)

7.0 M ammonium acetate (7.0 M NH_4OAc) pH 7.7

Sterile eppendorf tubes

Sterile distilled deionized water (ddH_2O)

Methods

Mini-prep for small scale DNA extraction

Extraction of the total genomic DNA from fresh, young leaf tissues were carried out using a modified CTAB method by Doyle and Doyle (1987).

First, 1.5 ml of CTAB extraction buffer was added in with 3 µl β-mercaptoethanol into a 1.5 ml Eppendorf tube, which was preheated to 65°C in a water bath for almost 30 minutes. Sufficient extraction buffer was added in, in order for easy dispersion of clumps though the solution remained viscous. Then, fresh young leaf tissue was ground to powder form in liquid nitrogen with a chilled mortar and pestle. In order to obtain high yield of high molecular weight DNA, the tissue was

ground to a very fine powder form in the liquid nitrogen and the tissues were kept frozen during this process.

Three or four scoops of finely ground powder were then scraped and were added in directly into preheated buffer and were swirled gently to mix. In order to minimize physical shearing of DNA, extreme care was practiced when mixing during the extraction steps. Violent shaking with a shaker or a vortex was avoided. Next, the sample was incubated at 65°C for 2 hours with possible occasional gentle swirling. Temperature throughout this step was kept above 15°C to prevent CTAB precipitation.

After the incubation period, 1 ml of the aqueous phase of the sample was transferred to a new Eppendorf tube and 500 µl of chloroform-isoamyl alcohol (24:1; v/v) was added in. The mixture was then thoroughly but gently mixed. Cell wall debris, denatured protein and most polysaccharides were removed simultaneously during extraction with chloroform-isoamyl alcohol (24:1; v/v). Next, the sample was centrifuged at 13000 rpm for 15 minutes in a non-refrigerated centrifuge (fixed angle rotor) at room temperature to separate the different phases.

The uppermost viscous aqueous phase was transferred to a clean centrifuge tube using a wide-bore pipette (blue tip), which was prepared by cutting off the pointed end of the tip. The white interface (two-protein precipitate) was not disturbed. A 2/3 volumes of cold isopropanol (-20°C) was added and then mixed gently to obtain nucleic acids precipitate. The sample was stored overnight.